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OBSERVATION OF COLLOIDAL GOLD LABELLED PLATELET SURFACE RECEPTORS AND THE UNDERLYING CYTOSKELETON USING HIGH VOLTAGE ELECTRON MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

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Abstract

Fibrinogen conjugated to colloidal gold or colloidal gold-monoclonal anti-glycoprotein IIb/IIIa (fibrinogen receptor) was used to label the receptor on platelets. Whole mount preparations were examined by stereo pair high voltage electron microscopy and then by scanning electron microscopy to determine the feasibility of this approach in detecting the number of receptors and their location relative to the cytoskeletal and surface structure. Both the ligand-gold and antibody-gold labels were effective. The relative numbers of receptors could be seen and their relationship to cytoskeletal structure could be determined. Marked differences in receptor number and distribution were observed when platelets in different stages of activation were compared. In co-cultured macrophages and platelets, receptors were found exclusively on platelets or on pieces of platelet membrane adherent to macrophages.

Key Words: Platelets, Macrophages, Receptors, Immunolabelling, Colloidal Gold, Cytoskeleton, High Voltage Electron Microscopy, Scanning Electron Microscopy, Fibrinogen, Surface Glycoproteins

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Introduction

Production of Gold Sols

The chemical production of colloidal gold was described as early as 1845 by Selmi (44). The English physicist, Michael Faraday, was apparently fascinated by the gold colloids and devoted a considerable amount of energy to their study. It is reported that his methods for stabilization were so refined that certain of the colloids he made over a century ago still exist in the colloidal state [11,35].

Colloidal gold is a monodisperse lyophobe (hydrophobe) colloidal suspension or sol. Its production from gold chloride by a variety of reducing agents, as well as ultrasonics, has been variously reported and reviewed [4,21,24,51,53,54]. Very briefly, nucleation of the gold occurs in conditions of supersaturation. It is felt that a burst of nuclei form, then grow to their ultimate size. Since, in most systems, nearly all the gold ends up in the reduced state, the size of the colloidal gold particles or "beads" depends on the number of original nuclei. The number of nuclei is, in turn, a function of the amount and/or type of reducing agent [14,51]. Thus bead size can be carefully controlled and beads can be produced in sizes ranging from <5 to >100 nm.

Stabilization of Gold Sols

Once formed, stability is maintained by a balance of electrostatic forces of repulsion, due to the net negative charge of the particles, and van der Waals electrodynamic forces of attraction [35,52]. The van der Waals forces decay as an inverse power of the distance, while the electrostatic repulsion decays exponentially with distance. The electrostatic forces maintain the particles at a sufficient distance from one another so that particle aggregation does not occur. However, if the electrically charged layer is compressed by the addition of positively charged ions, the distance between particles is reduced so that the forces of attraction cause an essentially irreversible aggregation [39,45,46]. When this aggregation occurs in suspension, a change in the absorptive properties occurs so that the color rapidly changes from the orange-red (exact color depends on bead size) of the colloid to a blue characteristic of the aggregate [18,19].

There are several approaches to stabilizing suspensions of colloidal gold; however, most pertinent to the making of gold labels for microscopy is coating of the individual particles with various proteins or polymers, such as polyethylene glycol. This provides a "steric repulsion" such that the beads are prevented from coming close enough to one another, even in the presence of positively charged ions, for aggregation to occur. Once stabilized in this fashion, the gold particles can be suspended in nearly any buffer and remain stable [21,35].

Conjugation of Protein to Colloidal Gold

The best coating of the gold particles with protein occurs with the pH at or very slightly above the isoelectric point of the protein. At this point, the protein is in the zwitter ion form and hence has no net charge, has the maximum surface tension, and is least soluble, hence weakly hydrated [36]. This situation favors attachment to the hydrophobic gold particles. Acidic to the isoelectric point, the protein is positively charged and the electrostatic interaction between the negatively charged gold and positively charged protein results in bead flocculation or aggregation. Alkaline to the isoelectric point, the protein has a negative charge, resulting in repulsion between itself and the bead, causing poor protein attachment. Under the appropriate conditions of pH and protein concentration, stabilization of the beads is achieved, the reactive sites of the protein are retained, and the protein binding is nearly irreversible [10,21]. In practice, to achieve these conditions, it is often necessary to construct pH and protein concentration isotherms [12,15,19,21]. Also of importance is the suspension of the protein in distilled water or a buffer concentration as low as possible but which will maintain the protein's solubility. The gold is added to the protein solution followed, in test preparations, by the addition of excess sodium chloride to check for stability. If stabilization is incomplete, the colloid will aggregate and change color; if stable, the colloid remains and no color change occurs. This color change can be followed visually or spectrophotometrically [15]. The activity of most proteins (antibodies, enzymes, ligands) remains good following their attachment to gold particles [5,18,42].

For a more extensive discussion of gold-protein attachment the reader is referred to several original and review articles on the subject [3,10,17,20,21,29,41,42,48,49].

Colloidal Gold and High Voltage Electron Microscopy

For our purposes the colloidal gold provides a sufficiently dense marker that is easily distinguishable with the high voltage electron microscope (1000 kV) and has a regular spherical shape which can be visualized in the scanning electron microscope [21]. Ligands for specific receptors or antibodies specific for the glycoproteins which compromise the receptor can be directly coupled to the beads with little or no loss of specificity [27].

The direct coupling permits the finest resolution of the receptor positioning on the cell surface, although antibody or antibody-protein A, or various applications of 2nd antibody may provide sufficient resolution depending on the information required [6,9,16,21,38,40,42,47]. However, whether direct or indirect labelling procedures are used, bead size must also be taken into account, as larger beads often result in a less dense labelling pattern (Figures 1 and 2).

Our primary goal has been the development of procedures which would permit visualization of the cell cytoskeleton and at the same time permit labelling of surface receptor glycoproteins. In this way the relationship (or lack of it) of the surface receptors to the underlying cytoskeleton could be visualized. The smaller size of platelets (1 μm diameter), their dynamic cytoskeleton, defined surface receptor sites, rapid activation and shape change sequence, as well as the absence of a nucleus, make this an excellent system [50]. Since the platelet fibrinogen receptor has been previously characterized as a glycoprotein IIb/IIIa complex and since both purified fibrinogen and monoclonal anti-IIb/IIIa are available, we utilized this system [7,13,23,31]. The high voltage electron microscope permits viewing of whole platelets or of whole platelet cytoskeletons prepared by detergent extraction of platelets [1,25,28,33]. Stereo imaging is extremely useful in determining the complex three dimensional structure of the cytoskeleton and the actual location of the gold label relative to the surface and underlying structures [26,27]. Scanning electron microscopy can be used to determine the surface distribution of the labels [16,26,34].

Materials and Methods

Preparation of Colloidal Gold

A stock solution of colloidal gold granules having an average diameter of 18 nm were prepared by reducing $HAuCl_4$ with trisodium citrate. A 4% solution of $HAuCl_4$ (0.5 ml) was added to 200 ml of deionized distilled water and brought to a boil. A freshly prepared solution of 1% trisodium citrate (5 ml) was rapidly added to the boiling solution and the mixture refluxed for 30 min. The formation of the monodisperse colloidal particles was indicated by a dark blue to red color change. The colloidal solution was cooled, filtered through a microporous filter (Millipore Millex-GS 0.22-μm filter unit), and stored at 4°C under sterile conditions.

Preparation of Fibrinogen, Fibrinogen-Gold, and Antibody-Gold Complexes

Fibrinogen, a gift of Dr. Deane Mosher, University of Wisconsin, was purified from fresh human citrated plasma by precipitation with 25% saturated ammonium sulfate followed by DEAE-cellulose chromatography [32]. The final product was dialyzed against 0.01 M Tris and 0.14 M NaCl, pH 7.4, and aliquots of 10-15 mg/ml were stored at -70°C.

Adsorption isotherms were performed to determine the minimum amount of fibrinogen necessary to stabilize the colloidal gold. Previous investigations have indicated that optimum adsorption occurs at or slightly basic to the pI of the protein. The gold solution was adjusted to pH 6.5 with 0.2 N K_2CO_3 as measured by gel-filled combination electrode (No. 9115, Orion Research, Inc., Cambridge, MA). A series of fibrinogen solutions of increasing concentration were made up

to 1 ml and added to 5 ml of colloidal gold. After 1 min at room temperature, 1 ml of 10% NaCl solution was added to the fibrinogen-gold. Inadequate stabilization of the colloids results in flocculation of the gold granules, with flocculation indicated by a color change from red to blue. The minimum amount of fibrinogen necessary to prevent flocculation was 8 μg/ml of gold solutions.

Ten ml of gold solution (pH 6.5) was added to a 10% excess of dialyzed fibrinogen (0.005 M NaCl) and mixed by gentle inversion. After 5 min at room temperature, 0.5 ml of freshly prepared and prefiltered (Millipore 0.45 μm) polyethylene glycol 20,000 mol wt was added to prevent aggregation. The fibrinogen-gold was concentrated and excess fibrinogen removed by centrifugation in polycarbonate tubes at 10,000 rpm for 30 min. The supernatant was discarded, and the concentrated red pool was suspended to 1 ml with sterile filtered (Millipore 0.20 μm) protein-free Tyrodes buffer supplemented with 1 mM Ca++.

The monoclonal antibody 10E5, which is directed against the glycoprotein IIb/IIIa receptor complex, was a gift from Dr. Barry Coller (State University of New York at Stonybrook Health Sciences Center). The specificity of 10E5 has been fully characterized in a previous report [8]. The minimum amount of antibody necessary to stabilize the gold particles was determined by adsorption isotherms as described above. After conjugation and centrifugation, the antibody-gold complex (10E5-Au) was resuspended to 1 ml with sterile filtered (Millipore 0.2 μm) 0.1 M phosphate buffer, pH 7.4.

Platelet Preparation and Gold Labeling

Platelets were obtained from normal healthy adult volunteers. Blood samples (10 ml) were collected in polypropylene tubes containing 10 mM EGTA and mixed by gentle inversion. Platelet-rich plasma was prepared by centrifugation of whole blood at 180 g for 10 min at room temperature. Platelets were separated from plasma proteins by passage through a Sepharose CL-2B column having a 40 ml bed volume. The column was equilibrated at room temperature with a calcium-free Tyrodes buffer, pH 7.3 (136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 1 g/liter dextrose and 2 g/liter albumin). Platelets were collected in the void volume and deposited on Formvar-filmed nickel Maxtiform grids and allowed to settle and adhere at 37°C in a moist chamber. The extent of spreading was monitored by phase-contrast microscopy, and at various stages of spreading, grids containing adherent platelets were removed and washed with protein-free buffer. The time frame for platelet spreading after surface activation has been previously well-documented [2]. From the time of contact with the Formvar film, the entire spreading process requires ~ 12-15 min. Maximum incubation time was 20 min.

Platelet cytoskeletons were prepared by previously described extraction procedures [1,43]. The extraction buffer, PHEM, consisted of 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9. Spread platelets were washed with PHEM buffer, then lysed with 0.15% Triton X-100 in PHEM buffer for 1 min. Grids were thoroughly washed in buffer and prepared for electron microscopy as described below.

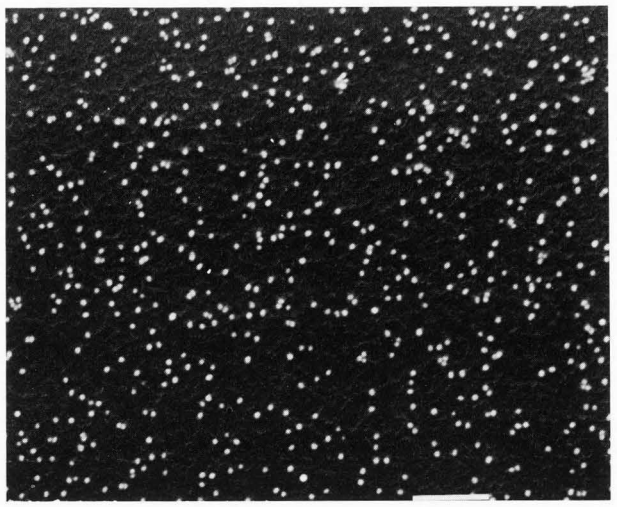


Figure 1. Secondary electron scanning electron micrograph of an albumin-coated polymer surface labelled with 30 nm colloidal gold particles conjugated to anti-albumin. Note the relative uniformity and density of labelling. Bar = 1.0 μm.

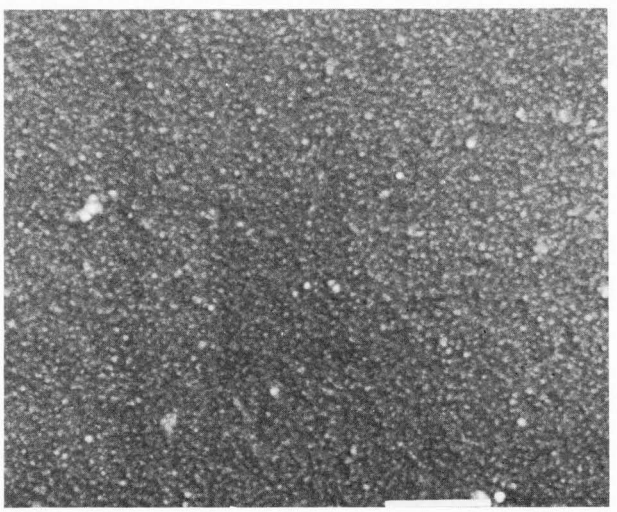


Figure 2. Secondary electron scanning electron micrograph of the same surface as in figure 1 except labelling is with much smaller 18 nm gold particles conjugated to anti-albumin. Labelling is still relatively uniform; however, the total number of labels has increased significantly. Bar = 1.0 μm.

Platelets were labelled by incubating individual grids in 20 μl of the FGN-Au or 10E5-Au suspension for 5 min at room temperature. In control experiments platelets were incubated with albumin-labelled colloidal gold, colloidal gold conjugated to the IgG fraction of normal serum, or with a large excess of soluble fibrinogen (2 mg/ml) before incubation in the FGN-Au suspension. Grids were thoroughly washed in buffer to remove unbound label and processed for electron microscopy.

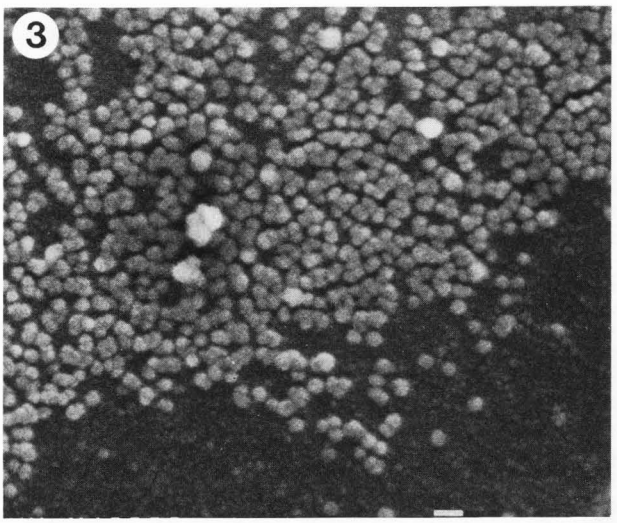
Electron Microscopy

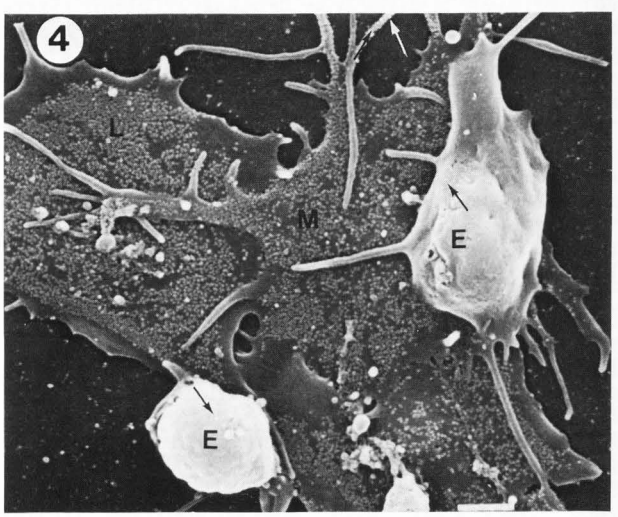
All samples were fixed in 0.1 M HEPES-buffered 1% glutaraldehyde, 0.05% tannic acid, 0.05% saponin, pH 7.3, for 30 min at room temperature (30). They were then rinsed in HEPES, post-fixed in HEPES-buffered 0.05% OsO_4 for 15 min., and stained in 1% UrMgAc (Aq.) for 15 min. Specimens were dehydrated through a graded series of alcohol to absolute alcohol, itself dried by storage over molecular sieve. Samples were dried from liquid CO_2 by the critical point procedure in a critical point dryer equipped with an in-line molecular sieve filter and a hydrophobic water-excluding filter (37). Samples were evaporatively coated with a thin layer of carbon and stored over molecular sieve until examined with the AEI EM 7 1 MeV electron microscope of the Madison HVEM facility. Stereo pair micrographs were taken at tilt angles appropriate for specimen thickness and magnification (22). Some samples were evaporatively or sputter coated with ~ 10 nm of gold or gold-palladium and examined on a JEOL JSM 35C scanning electron microscope at 10-20 kV accelerating voltage. A tilt angle of 7° was used for SEM stereo pairs.

The number of FGN-Au labels bound per platelet at saturation was determined by direct counting of labels on individual platelets. Micrographs of randomly selected well spread platelets from three separate experiments were enlarged to a final print magnification of 100,000. Individual labels were counted and marked on a transparent overlay.

Results

The gold beads conjugated to fibrinogen are readily visible on the cell surface when viewed by scanning electron microscopy (Figure 3). Individual labels can be identified. The overall distribution of the receptor for fibrinogen can be seen and differences in distribution due to the stage in activation can be compared (Figure 4 and 5). The internal structure of unlabelled platelets is visible by stereo pair HVEM and the various zones characteristic of the different morphologic stages in the platelet shape change response can be





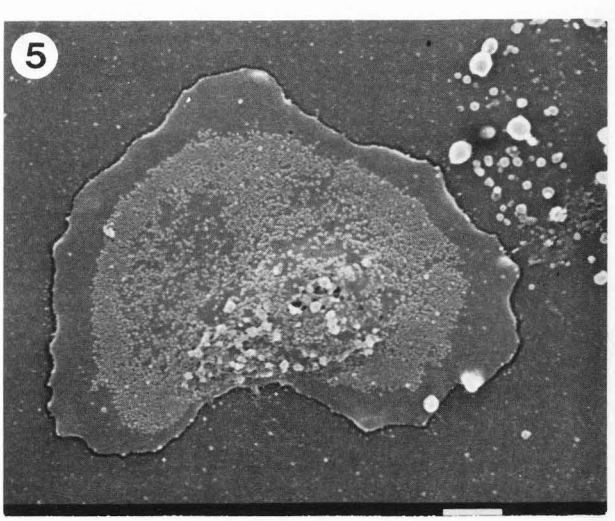


Figure 3. 18 nm colloidal gold conjugated to fibrinogen has been used to label the glycoprotein IIb/IIIa fibrinogen receptor on the platelet surface. Individual labels can be readily identified by SEM. Bar = 0.1 μm

Figure 4. Several platelets in different stages of activation. The early, more rounded, stage cells (E) show minimal presence of the active fibrinogen receptor (arrows) as detected by the 18 nm gold-fibrinogen beads. The mid-activation, pseudopodial forms (M) demonstrate extensive binding of the gold-fibrinogen over the entire surface of the platelet including the pseudopods (arrows). The late stage cell (L) is more completely spread, has fewer pseudopodia, and receptors have moved toward the center of the cell leaving a label-free zone at the cell periphery. Bar = 1.0 μm.

Figure 5. A fully spread platelet labelled with fibrinogen-gold. The inward movement of the receptors in the plane of the membrane results in the clearly identifiable receptor-free zone at the cell margins. Bar = 1.0 μm.

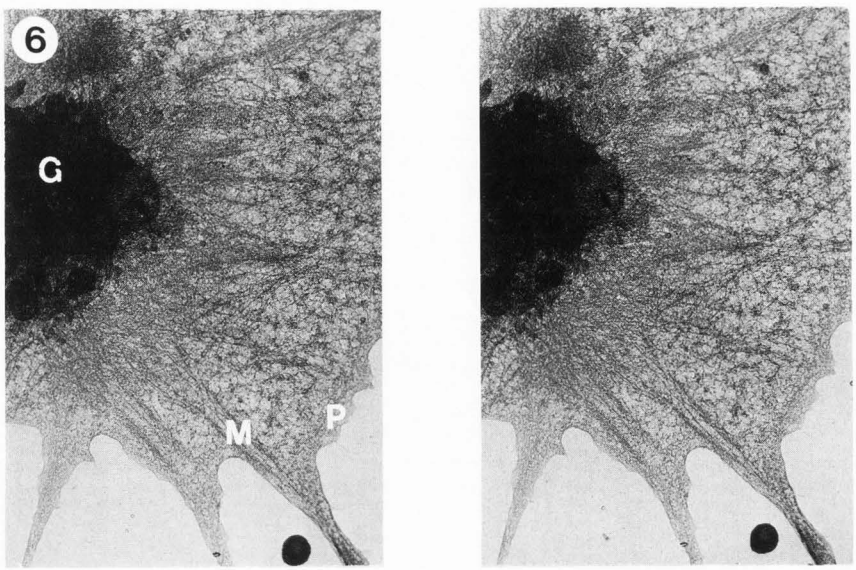


Figure 6. Whole mount HVEM stereo pair demonstrating the internal organization seen in the pseudopodial stage of non-detergent extracted platelets. Microfilament bundles (M) can be seen to radiate from the granulomere area (G) into the pseudopods. The marginal peripheral web (P) can also be identified. Labelling of cells at this stage demonstrates presence of active fibrinogen receptors over the entire platelet surface including the pseudopods. Picture width = 3.8 μm.

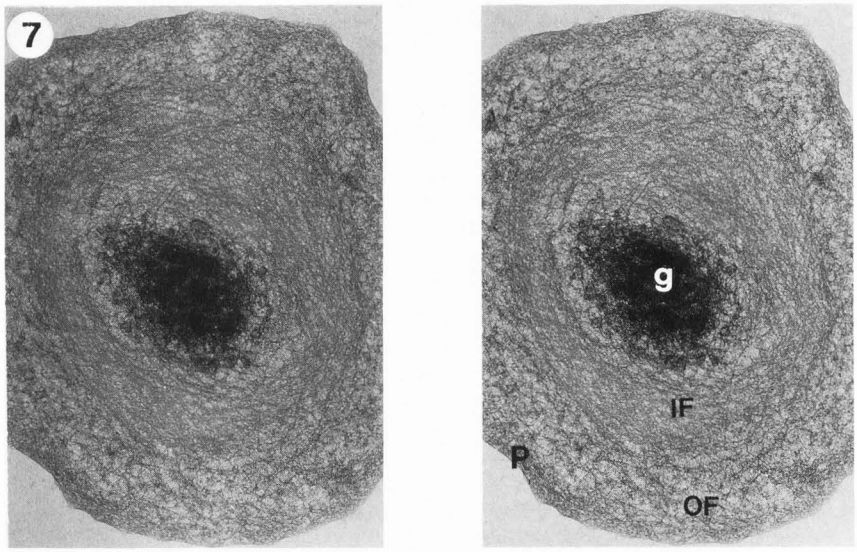


Figure 7. Stereo pair HVEM demonstrating the internal organization of a fully spread platelet. The peripheral web (P), outer filamentous zone (OF), inner filamentous zone (IF) and granulomere (g) can be seen. Picture width = 3.8 μm.

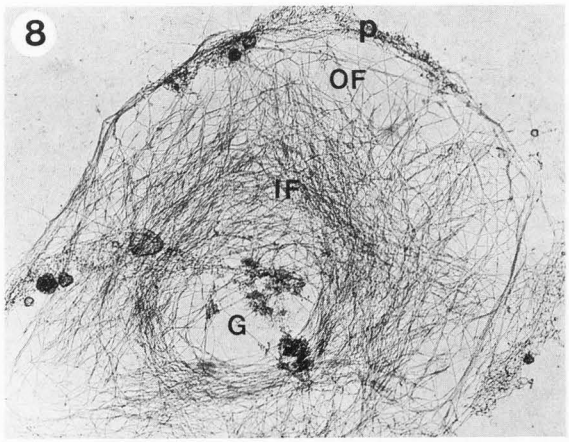


Figure 8. HVEM of a detergent extracted, fully spread platelet. Only cytoskeletal elements, principally actin filaments, remain. The relationship of the cytoskeleton to the zones can be seen. The four zones, peripheral web (P), outer filamentous zone (OF), inner filamentous zone (IF), and the granulomere area (G), can be identified. The granules have been extracted hence the area is relatively "open" as only the supporting lattice of microfilaments remains. The cytoskeleton of the inner filamentous zone is seen to be composed of a dense actin filament network. Scanning electron micrograph of similar preparations show the filaments to be arranged in a "basket-weave" pattern. The outer filamentous zone has a more open microfilament network and many filament-to-filament connections can be identified. Pseudopods and associated microfilament bundles are absent at this stage. Picture width = 4.5 μm.

identified (Figures 6 and 7). Detergent extraction of whole platelets permits a more detailed examination of the cytoskeletal elements underlying the specific zones (Figure 8).

Stereo pair HVEM of unextracted labelled platelets using either fibrinogen-gold or anti-IIb/IIIa-gold (antibody specific for the receptor glycoprotein complex) defines the location of the fibrinogen receptors and clearly identifies their distribution relative to the underlying cytoskeletal elements (Figures 9 and 10). Differences in both the receptor distribution and the cytoskeletal organization from stage to stage can be compared. The specificity of the labelling is demonstrated in Figure 11. In this case platelets and macrophages have been allowed to settle on the Formvar films. Macrophages, which apparently lack the IIb/IIIa complex on their surface, are unlabelled by monoclonal antibody specific for platelet IIb/IIIa while the platelets are clearly labelled. This is seen to be the case even when platelets are directly attached to macrophages.

Soluble antibody or ligand added prior to gold-ligand or gold-antibody effectively blocks all binding of the gold-labelled ligand or antibody, respectively. No label is seen when platelets are labelled with albumin-Au or with gold conjugated to the IgG fraction of normal serum.

Conclusion

The density of colloidal gold beads permits them to be readily identified by HVEM. Their regular shape, in addition to composition, allows them to be viewed with SEM. They can be used to localize cell surface receptor sites either by complexing them to ligand specific for the receptor site or by coupling them to antibody specific for the glycoproteins which comprise the receptor site. Stereo pair HVEM of whole cell preparations can be used to simultaneously view both receptor distribution and the internal ultrastructure, especially that of the cytoskeleton. Such studies should facilitate comparison of cell surface receptor distribution and movement with changes in the organization of the internal cell structure both in platelets and nucleated cells.

The use of directly coupled ligand or monoclonal antibody permits a precise spatial resolution of receptor or antigen distribution. With the smaller gold bead sizes, for example, the size of the bead and attached antibody or ligand (7 to 10 nm) is roughly the size of the reported Stokes radius of the receptor complex. Techniques employing gold coupled to secondary antibody or protein A can also be very effective; however, some loss of spatial resolution is to be expected.

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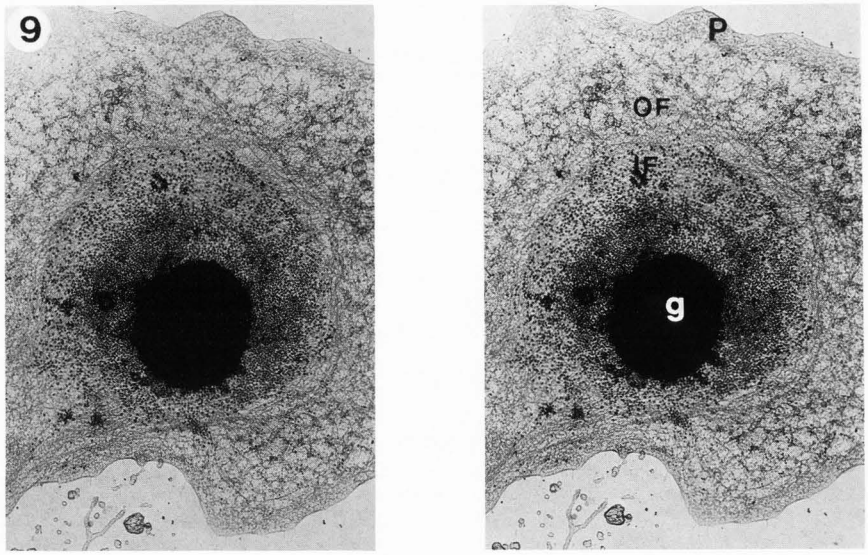

Figure 9. Stereo pair HVEM of an unextracted, fully spread platelet. The cell is in the late stage of activation. Fibrinogen receptors have been labelled with 18 nm gold-fibrinogen. The four zones seen in fully spread platelets are apparent: (P), peripheral web; (OF), outer filamentous zone; (IF), inner filamentous zone; and (G), granulomere. The inward movement of the fibrinogen receptor is complete and as can be seen, their distribution corresponds exactly with the subjacent inner filamentous zone. The outer margin of the labels coincides with the outer edge of the inner filamentous zone. Picture width = 4.0 μm.

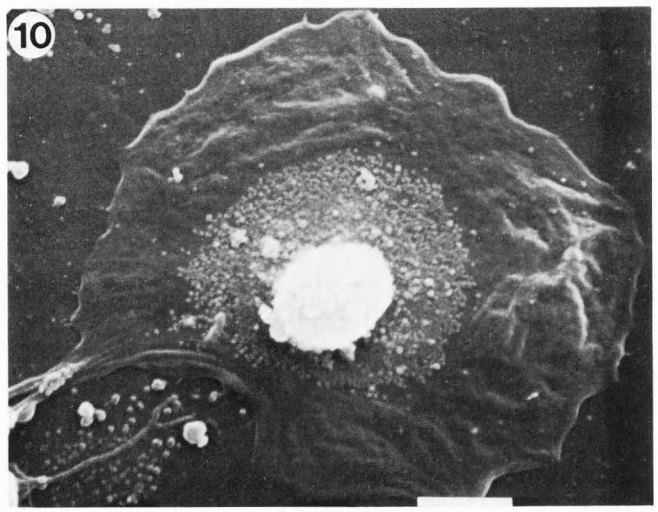

Figure 10. SEM of the same platelet as in figure 9. The localization of the receptors can be seen relative to the surface of the cell. The underlying cytoskeletal zones are not apparent on the SEM; however, the central position of the receptors on the surface of the platelet is clear. Bar = 1 μm.

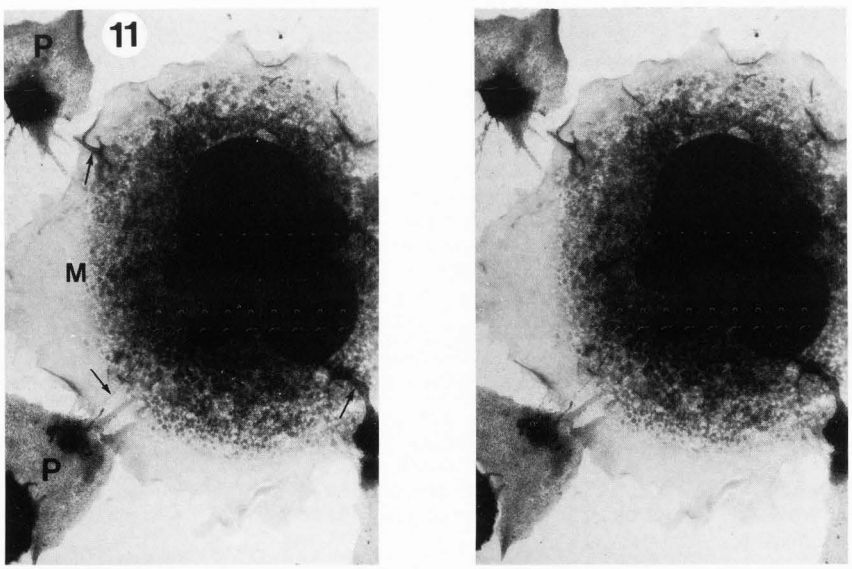

Figure 11. HVEM stereo pair of an adherent macrophage and adjacent platelets. The cells have been incubated with monoclonal anti GpIIb/IIIa (anti-fibrinogen receptor antibody) - gold. The platelets (P) demonstrate dense labelling while the macrophage (M) shows no labelling. Areas where platelets or platelet pseudopods are in contact with the macrophage (arrows) show label on the platelet membranes but no label on or in the macrophage. Thus the macrophage appears to lack the GpIIb/IIIa and reports of its presence on macrophages could be due to a contaminating population of platelets. In addition, whole platelets or platelet membranes are often seen to be adherent to macrophages prepared from peripheral blood. Picture width = 17 μm.

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